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Enhanced Cellular Oxidant Stress by the Interaction of Advanced Glycation End Products with Their Receptors/Binding Proteins*

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Attack by reactive oxygen intermediates, common to any kinds of cell/tissue injury, has been implicated in e development of diabetic and other vascular diseases. ich oxygen-free radicals can be generated by advanced ycation end products (AGEs), which are nonenzymati- lly glycated and oxidized proteins. Since cellular in- ractions of AGEs are mediated by specific cellular nding proteins, receptor for AGE (RAGE) and the ctoferrin-like polypeptide (LF-L), we tested the hy- thesis that AGE ligands tethered to the complex of AGE and LF-L could induce oxidant stress. AGE albu- in or AGEs immunoisolated from diabetic plasma re- lted in induction of endothelial cell (EC) oxidant ess, including the generation of thiobarbituric acid active substances (TBARS) and resulted in the activa- n of NF-κB, each of which was blocked by antibodies AGE receptor polypeptides and by antioxidants. Infu- n of AGE albumin into normal animals led to the ap- arance of malondialdehyde determinants in the vessel ll and increased TBARS in the tissues, activation of -κB, and induction of heme oxygenase mRNA. AGE- duced oxidant stress was inhibited by pretreatment of imals with either antibodies to the AGE receptor/ nding proteins or antioxidants. These data indicate at interaction of AGEs with cellular targets, such as s, leads to oxidant stress resulting in changes in gene pression and other cellular properties, potentially ntributing to the development of vascular lesions. rther studies will be required to dissect whether oxi- nt stress occurs on the cell surface or at an intracel- ar locus.

When proteins are incubated with ketoses, they undergo nenzymatic glycation and oxidation (1–3). The ultimate re- t of these interactions is a class of modified proteins termed anced glycation end products (AGEs).[1] This heterogeneous group of glycated structures is found in the plasma and accumulates in the vessel wall and tissues during aging and at an accelerated rate in diabetes (4, 5). Their presence has been hypothesized to contribute to the development of diabetic complications, such as accelerated atherosclerosis and microvascular disease.

One mechanism through which AGEs exert their cellular effects is through interaction with specific cell surface binding proteins (6–9). We have recently characterized endothelial cell (EC) and mononuclear phagocyte (MP) receptors for AGEs (7–9). This cellular binding site for AGEs consists of a novel integral membrane protein in the immunoglobulin superfamily, termed receptor for AGE (RAGE), and a lactoferrin-like polypeptide (LF-L) which is highly homologous/identical with lactoferrin (LF). LF-L associates in a high affinity, noncovalent fashion with RAGE, and we hypothesize that these two polypeptides comprise the endothelial cell binding site (10).

In view of the established association of glycation and oxidation (2, 11, 12), including generation of reactive oxygen intermediates (ROIs) induced by glycated proteins (13, 14), we hypothesized that binding of AGEs to their cellular receptors, such as those on ECs, would result in generation of an oxidant stress capable of altering gene expression and cellular properties in a manner such as to promote the development of vascular lesions. Our results demonstrate that interaction of AGEs with their cell surface binding sites leads to oxidant stress, manifested by the appearance of malondialdehyde determinants in the vessel wall, thiobarbituric acid-reactive substances in the tissues, induction of the transcription factor NF-κB, and induction of heme oxygenase mRNA. Taken together, the results of our *in vitro* studies with cultured capillary ECs and our *in vivo* infusion studies indicate that the interaction of AGEs with their cellular receptors/binding proteins localizes induction of ROIs to the vessel wall and is, we propose, likely to contribute to the oxidant stress which has been hypothesized to underlie diabetic vascular disease (3, 15, 16).

MATERIALS AND METHODS

Purification of Proteins, Preparation of Antibodies and Other Reagents—Bovine serum albumin (BSA; Sigma) was glycated by incubation with glucose (0.5 M) or glucose 6-phosphate (0.5 M) at 37 °C for 6 weeks or the indicated time. Glycated proteins were characterized based on fluorescence, binding to cultured ECs, MPs, and to purified RAGE (6–9). Controls consisted of the same initial preparations of albumin incubated at 37 °C in the same manner, except no ketose was present. Other glycated proteins were prepared similarly, including AGE immunoglobulin fraction (AGE IG; this was a 50% ammonium sulfate saturation precipitate from rat serum resuspended in and extensively dialyzed *versus* 0.02 M Tris, pH 7.4/0.1 M NaCl), AGE bovine prothrombin (purified prothrombin was obtained from Enzyme Re-

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The abbreviations used are: AGE, advanced glycation end products; GE, receptor for AGE; LF-L, lactoferrin-like polypeptide; EC, endo- ial cell; TBARS, thiobarbituric acid reactive substances; MP, mono- lear phagocyte; ROI, reactive oxygen intermediate; BSA, bovine serum albumin; IG, immunoglobulin; TNF, tumor necrosis factor; LDL, low density lipoprotein; EMSA, electrophoretic mobility gel shift assay; NI, nonimmune.

search Laboratories, South Bend IN), poly-L-lysine (38 kDa; Sigma), AGE fibronectin (purified fibronectin was provided by the New York Blood Center, NY), and AGE keyhole limpet hemocyanin (Sigma). Where indicated, preparations of AGE albumin were treated with $NaBH_4$ using a 200-fold excess of reducing agent for 1 h at room temperature (17).

Albumin was formylated or maleylated as described by Horiuchi *et al.* (18) and Haberland and Fogelman (19), respectively. Human LDL, acetylated LDL, and oxidized LDL were generously provided by Drs. Deckelbaum and Tabas (Depts. of Pediatrics and Medicine, Columbia).

Bovine AGE binding proteins, RAGE and LF-L, were purified to homogeneity (7), and monospecific antisera to each polypeptide were prepared and characterized as described (7, 9, 20). Anti-RAGE IgG and anti-LF-L Ig each separately blocked the binding of ^{125}I-AGE albumin (prepared by the lactoperoxidase method (21) to ECs or MPs and to purified RAGE, as described (7, 9).

Bovine LF was purified to homogeneity (22; generously provided by Ms. Hegarty and Dr. Hurley, Division of Animal Sciences, Univ. of Illinois, Urbana), and, for certain experiments, LF was obtained from Sigma, with identical results. Rabbit anti-bovine LF was prepared in rabbits (22), and the IgG was purified on immobilized protein A. Apolactoferrin and iron-loaded LF were prepared as described (10).

Superoxide dismutase (from bovine liver), catalase, glutathione peroxidase, and cytochrome *c* (horse type III) were purchased from Sigma, and desferoxamine was purchased from CIBA Pharmaceutical Co. (Summit, NJ). Mouse thrombomodulin, purified to homogeneity by the procedure used for rabbit thrombomodulin (23), was employed as immunogen for rabbits.

Preparation of Anti-AGE Antibody and Assays for AGEs in Diabetic Sera—Polyclonal antibody which selectively recognized AGE-modified proteins was prepared by immunizing guinea pigs with AGE albumin or AGE keyhole limpet hemocyanin using standard methods (24). IgG was purified by chromatography on protein A-Sepharose CL-4B (Pharmacia; Ref. 25), was adsorbed with native albumin Affi-Gel 15 (Bio-Rad) when the immunogen was AGE albumin (there was no residual immunoreactivity with native albumin; see below), and was then affinity-purified on an AGE albumin-Affi-Gel 15 column. Adsorbed IgG was eluted with glycine buffer (pH 2.5), and antibodies were immediately neutralized and then dialyzed against phosphate-buffered saline (pH 7.4). Assays for AGEs in plasma were performed on samples from diabetic patients in the clinic at Columbia-Presbyterian Medical Center or from normal individuals (ages 25–42 years) after obtaining informed consent, according to a protocol approved by the Institutional Review Board. Total AGE antigen in citrated plasma (final concentration, 0.39% citrate) was determined by radioimmunoassay. ^{125}I-AGE albumin (0.01 ml), patient plasma (0.05 ml), and anti-AGE IgG (0.01 ml; 1.3 μg/ml) were incubated in assay buffer (0.06 ml; Tris/HCl, 0.01 M, pH 7.4; NaCl, 0.15 M; Nonidet P-40, 0.1%) for 18 h at 4 °C, and then protein A-bearing, formalin-fixed *Staphylococcus* A suspension (0.05 ml; 10% suspension; IgGSorb, Enzyme Center, Malden MA) was added for 30 min at 25 °C. The mixture was centrifuged (5 min at 10,000 rpm), and the pellet was washed once with assay buffer, centrifuged, and counted.

AGEs were immunoisolated from diabetic patient plasma by applying it to a column with immobilized affinity-purified anti-AGE antibody on Affi-Gel 10. The column was washed extensively with buffer and eluted with high salt (NaCl, 2 M), as described previously (9). For controls, the same volume of normal plasma was processed in the same manner. Following dialysis of the high salt eluates, they were assayed for AGE content as described above. To study the possible presence of AGE IgG, human IgG from normal and diabetic plasma was purified by chromatography on protein A (25). Purified IgG showed a single band on nonreduced SDS-polyacrylamide gel electrophoresis (26) and was studied for AGE-immunoreactive material as described above.

Cell Culture—Bovine adrenal capillary endothelial cells were isolated and characterized as described previously (7).

Assays for ROIs—The cytochrome *c* assay was performed in phosphate buffer (10 mM; pH 7.8) by incubating AGE-modified protein(s) with iron-loaded LF or apolactoferrin and cytochrome *c* (10 μM) in the presence of EDTA (100 μM) for 10 min at 25 °C, and increased adsorption at 550 nm was determined as described (14). Where indicated, anti-LF-L IgG, anti-LF IgG, or anti-AGE IgG was preincubated with either LF or AGE, respectively, for 2 h at 37 °C, followed by centrifugation. Assays were performed on the supernatants (similar results were obtained when samples were not subjected to centrifugation). Control experiments in which the same concentration of nonimmune rabbit IgG replaced antibodies to AGE binding proteins demonstrated no effect on generation of radicals in the cytochrome *c* reduction assay.

Evidence of oxidant stress was also detected by measuring generation of TBARS during the incubation of AGE-modified proteins with ECs (0.5–1.0 × 10^6 cells/assay) or from the organs of animals infused with AGE albumin. EC cultures were washed twice with Hank's balanced salt solution and then incubated for 18 h at 37 °C in serum-free minimal essential medium-α with no iron (Life Technologies, Inc.) containing the indicated concentration of native albumin, AGE albumin, or patient-derived AGEs. For determination of TBARS in cultured ECs, the method described by Dennery *et al.* (27) was employed. Where indicated, anti-RAGE IgG, anti-LF-L IgG, anti-LF IgG, nonimmune IgG (each of these antibodies was present at the concentrations indicated in the figures), probucol (50 μM), or *N*-acetylcysteine (30 mM) was added to the incubation mixture as follows: cultures were preincubated with anti-AGE binding protein IgG or nonimmune IgG for 1 h (37 °C) and washed, and fresh medium was added along with AGEs. In other assays, cultured ECs were preincubated for 16 h and 1 h with probucol or *N*-acetylcysteine, respectively, and then AGEs were added. For certain experiments, either superoxide dismutase, catalase, or glutathione peroxidase was added to EC cultures just prior to addition of AGEs.

For infusion studies, mice (CD1) were pretreated with either antibodies or antioxidants and then received either AGE albumin (100 μg/animal; intravenous) or native albumin (100 μg/animal; intravenous). In experiments with antioxidants, probucol (50 μM; intravenous) or *N*-acetylcysteine (30 mM; intravenous) was infused 0.5 h prior to AGE albumin. In studies with antibodies, either anti-RAGE IgG, anti-LF-L IgG, anti-thrombomodulin IgG, or nonimmune IgG (40 μg/animal in each case; intravenous) was administered 0.5 h before AGE or native albumin infusion. At the indicated times after the infusion study, animals were sacrificed, and organs were washed to remove blood with ice-cold saline, weighed, homogenized, and processed for TBARS determination by assessing changes in adsorbance at 532 nm. A standard curve was generated using dilutions of 1,1,3,3-tetramethoxypropane (Sigma). AGE albumin was nonreactive when tested in these assays for TBARS.

Northern Analysis for Heme Oxygenase and Activation of the Transcription Factor NF-κB—Mice (CD1) were pretreated with either antibodies to AGE binding proteins (anti-RAGE IgG, anti-LF-L IgG, or nonimmune rabbit IgG, as above) or probucol (as above) followed by intravenous infusion with AGE albumin (100 μg/animal) or native albumin (100 μg/animal). After 1 h, animals were sacrificed, and livers were excised. RNA was prepared by the guanidinium thiocyanate procedure, applied to agarose formaldehyde gels (0.8%; 30 μg/lane), and, following electrophoresis, transferred to nylon filters. Filters were prehybridized for 1 h at 68 °C with QUIK Hyb buffer (Stratagene) and hybridized in QUIK Hyb buffer for 3 h at 68 °C in the presence of ^{32}P-labeled murine heme oxygenase cDNA (28; generously provided by Dr. S. Sakiyama, Chiba Cancer Center Research Institute, Chiba, Japan), labeled by the random primer procedure. Filters were then washed with SSC (2×) containing SDS (0.1%) for 30 min at 55 °C, dried, and subjected to autoradiography. To assess RNA loading, filters were also hybridized with random primer labeled [^{32}P]cDNA for glyceraldehyde phosphate dehydrogenase (the cDNA for glyceraldehyde phosphate dehydrogenase was generously provided by Dr. Rick Assoian, Dept. of Biochemistry, Columbia Univ.) using the same wash and hybridization procedure.

Nuclear extracts were prepared from ECs (10^7 cells) exposed for 4 h at 37 °C to AGE albumin or native albumin (100 μg/ml, in each case) by the method of Dignam *et al.* (29). Where indicated, cultures were pretreated for 3 h with anti-RAGE IgG, anti-LF-L IgG, or nonimmune IgG (120 μg/ml) or for 1 h with probucol (50 μM) or *N*-acetylcysteine (30 mM). Certain cultures were exposed to purified human recombinant tumor necrosis factor (TNF; 10 ng/ml; generously provided by Knoll Pharmaceuticals, Whippany, NJ). Nuclear extracts were also prepared from the livers of animals infused with AGE or native albumin (100 μg/animal; intravenous). Where indicated, animals were pretreated with anti-RAGE IgG or anti-LF-L IgG (40 μg/animal in each case) for 30 min prior to the AGE albumin infusion. In other experiments, mice received probucol (50 μM) 1 h before the glycated protein infusion or only TNF (1 μg/animal). Complementary 27-base pair oligonucleotide probes representing the consensus murine NF-κB site (30) were as follows: 5'CCC CAG AGG GGA CTT TCC GAG AGG CTC3' (NF-κB1#) and 5'GGG GAG CCT CTC GGA AAG TCC CCT CTG3' (NF-κB2#). Oligonucleotides were annealed and 3' end-labeled with [α-^{32}P]dCTP and dGTP using Klenow polymerase via standard procedures. Binding reactions were performed by preincubating nuclear extract-protein (mouse liver nuclear extract, 11.4 μg; endothelial cell extract, 0.4 μg) in HEPES (20 mM; pH 7.9), KCl (60 mM), $MgCl_2$ (1 mM), EDTA (0.1 M), glycerol (10%), dithiothreitol (0.5 mM), poly(dI-dC) (2 μg) on ice for 10 min, followed by addition of the double-stranded ^{32}P-labeled oligonucleotide (0.2 ng) and

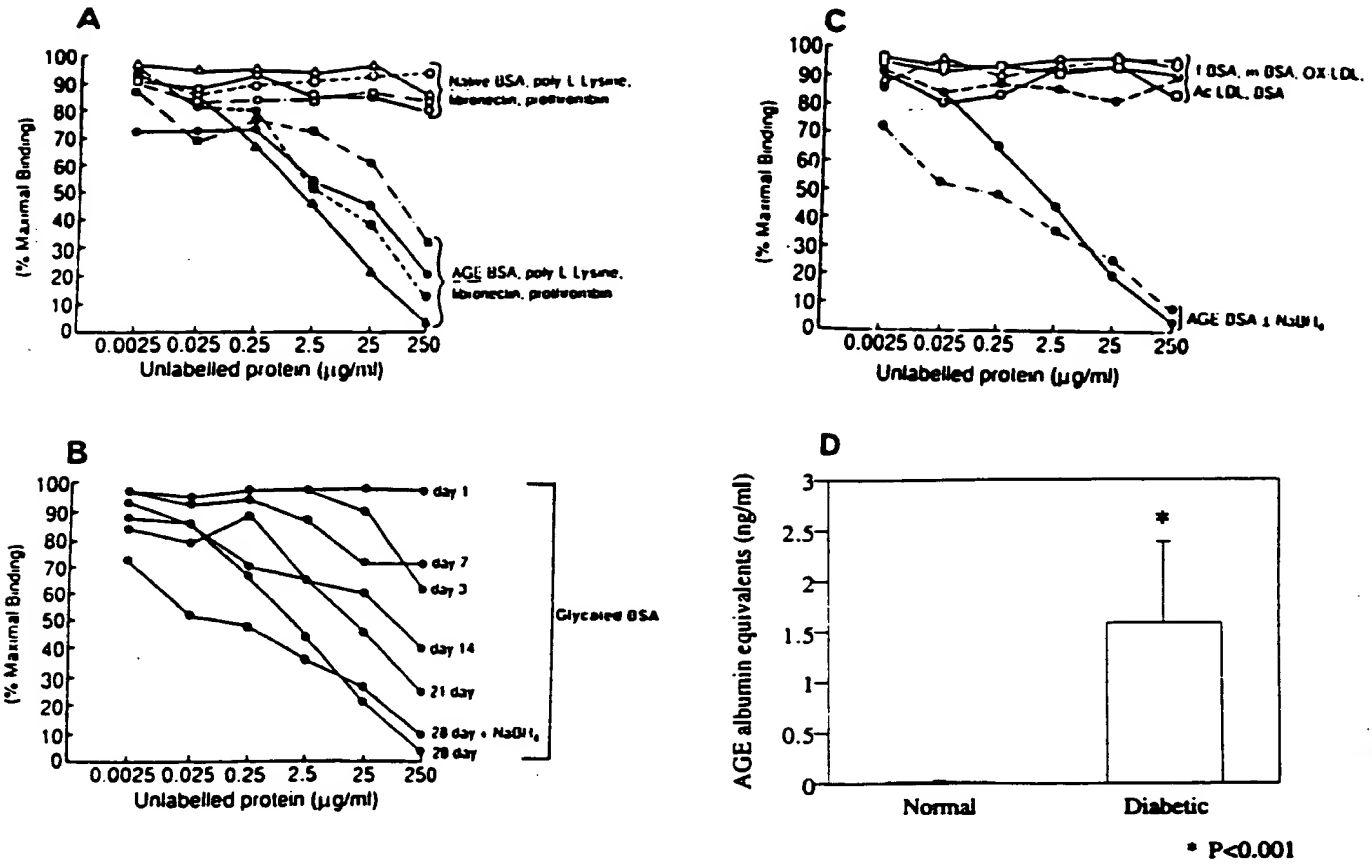

FIG. 1. **Specificity of anti-AGE antibodies and radioimmunoassay for AGEs in diabetic plasma.** *A*, affinity-purified anti-AGE antibodies were incubated with ^{125}I-AGE albumin in the presence of the indicated concentrations of unlabeled AGE-modified poly-L-lysine (*solid circle*), fibronectin (*solid circle, dotted line*), albumin (*BSA; solid triangle*) or prothrombin (*solid square*), or the native counterparts of these proteins (same symbol not filled in). The radioimmunoassay was performed as described in the text. Percent maximal binding (100% is that observed in the absence of unlabeled competitor) is plotted *versus* concentration of unlabeled competitor added. *B*, the same assay using anti-AGE antibody and ^{125}I-AGE albumin was performed in the presence of albumin prepared by exposure to glucose 6-phosphate for the indicated number of days. *C*, the same assay using anti-AGE antibody and ^{125}I-AGE albumin was performed in the presence of the indicated concentration of either unlabeled native bovine serum albumin (*BSA; triangle, dotted line*), AGE albumin (*AGE BSA – $NaBH_4$; closed circle, solid line*), AGE albumin pretreated with $NaBH_4$ (*AGE BSA + $NaBH_4$; closed circle, discontinuous line*), formylated albumin (*f-BSA; open square*), maleylated albumin (*m-BSA; open triangle*), oxidized LDL (*OX-LDL; closed circle, dotted line*), or acetylated LDL (*Ac-LDL; open circle, solid line*). *D*, radioimmunoassay for AGEs performed on samples of human diabetic (n = 25) and normal (n = 17) plasma. Results are expressed in terms of AGE albumin equivalents, based on a standard curve made with dilutions of AGE albumin prepared by incubating human albumin with glucose 6-phosphate.

a second incubation at room temperature for 20 min. Samples were loaded directly onto nondenaturing 6% polyacrylamide gels (29:1 acrylamide to bisacrylamide) prepared in Tris (45 mM)-boric acid (45 mM)-EDTA (0.1 M)-TBE (0.5×). Electrophoresis was performed at room temperature for 3–4 h at 15 mA. Gels were then dried and exposed to Kodak XRP film with intensifying screens. For competition studies, unlabeled probe for Sp1 (31) was also utilized: 5'GCT CCA GGC GGG GGC GGG GCC CGG GTT CGG3' (Sp1–1#) and 5'CCG AAC CCG GGC CCC GCC CCC GCC TGG AGC3' (Sp1–2#). Experiments with unlabeled oligonucleotides, either Sp1 or NF-κB, employed a 100-fold molar excess relative to the radiolabeled oligonucleotide.

Immunohistology for Malondialdehyde Determinants in Rats Infused with AGE Albumin—Rats (Sprague-Dawley) on a normal diet were anesthetized and infused with AGE albumin or native albumin (1 mg/animal), sacrificed at 1 h by perfusion with paraformaldehyde (4%)/sucrose (5%)/butylated hydroxytoluene (50 μM)/EDTA (1 mM) (final pH 7.4), and processed as described (32). Immunohistochemical staining was performed in the avidin-biotin-alkaline phosphatase system using murine monoclonal antibody to malondialdehyde (32) as the first antibody (20 μg/ml; this antibody was generously provided by Dr. Joseph Witztum, UCSD). Sites of antibody binding were detected using biotinylated goat anti-murine IgG and Extra Avidin alkaline phosphatase (Sigma). Controls were performed using nonimmune murine IgG.

RESULTS

AGE Albumin and AGE Immunoglobulin as Models of AGEs in the Plasma of Patients with Diabetes—To determine if AGEs prepared by incubating proteins with either glucose or glucose 6-phosphate *in vitro* provide a relevant model for AGEs which form *in vivo*, an antiserum selective for AGEs was developed to detect and to immunoisolate AGEs from diabetic plasma. Animals were immunized with AGE albumin prepared *in vitro*, antibodies in sera reactive with native albumin were removed by adsorption using native albumin-Affi-Gel, and the remaining antibody population was affinity-purified on AGE albumin Affi-Gel. Antibody eluted from the latter column selectively recognized the glycated forms of albumin, fibronectin, poly-L-lysine, and prothrombin (Fig. 1*A*), and preferentially reacted with those preparations of glycated albumin which contained advanced products (Fig. 1*B*); *i.e.* those reaction mixtures in which albumin and glucose 6-phosphate had been incubated for longer times (14, 21, or 28 days) rather than shorter intervals (1, 3, or 7 days). Consistent with this evidence that antibody was reacting with AGEs, sodium borohydride reduction f glycated albumin preparations did not significantly affect their immunoreactivity (Fig. 1*B*). Compared with albumin modified by other methods (formylation or maleylation) or modified LDL (oxidized or acetylated LDL), the anti-AGE antibody only reacted with glucose/glucose 6-phosphate-treated albumin (Fig. 1*C*). Similar results were obtained with antibodies prepared to

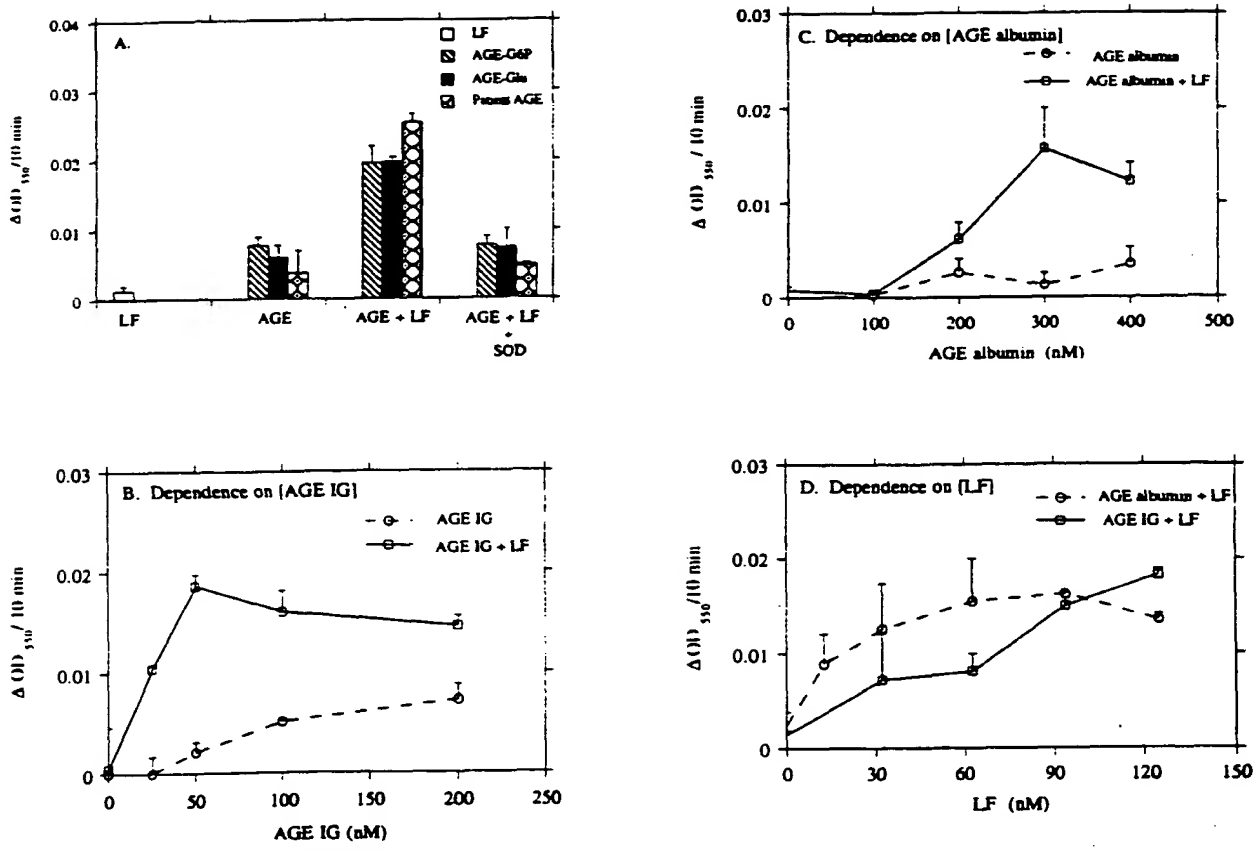

FIG. 2. **Generation of reactive oxygen intermediates by AGE-modified proteins: role of LF.** *A*, AGE albumin prepared *in vitro*, with either glucose 6-phosphate (*AGE-G6P*; 300 nM) or glucose (*AGE-Glu*; 300 nM), or immunoisolated from diabetic plasma (*patient AGE*; 80 nM AGE albumin equivalents) was incubated in buffer alone or in the presence of iron-loaded LF (0.125 μM). Where indicated, either LF or AGE were present alone (*LF* or *AGE*) or the complete incubation mixture (*AGE + LF*) was supplemented with superoxide dismutase (20 μg/ml; *AGE + LF + SOD*). Generation of ROIs was assessed in the cytochrome *c* reduction assay. *B*, the indicated concentration of AGE IG (glycated immunoglobulin fraction) was incubated in buffer alone or with LF (125 nM), and generation of ROIs was measured in the cytochrome *c* reduction assay. *C*, the indicated concentration of AGE albumin was incubated in buffer alone (*AGE IG*) or with LF (125 nM; *AGE IG + LF*), and generation of ROIs was studied. *D*, AGE albumin (300 nM) or AGE IG (100 nM) was incubated in the presence of the indicated concentration of iron-loaded LF, and generation of ROIs was studied. In each case, the mean ± S.E. of triplicate determinations is shown.

albumin or other proteins (such as keyhole limpet hemocyanin) glycated with either glucose or glucose 6-phosphate (data not shown). These immunologic reagents, similar to antibodies recently reported by two groups (33, 34), were used to detect AGEs (Fig. 1*D*) in plasma samples, where levels in diabetic (n = 25) plasma appeared to be >10-fold greater than levels in plasma from normal individuals (n = 17).

Since IgG has a relatively long half-life in the plasma (~23 days) (35), we hypothesized that it could undergo advanced glycation in the circulation in patients with diabetes, accounting for a portion of immunoreactive AGEs in diabetic plasma. IgG purified from normal/diabetic plasma on protein A-Sepharose consisted of a single major band with M_r ~ 150,000 on nonreduced SDS-polyacrylamide gel electrophoresis and was immunoreactive with anti-AGE IgG (data not shown). In contrast, IgG similarly prepared from normal, age-matched individuals was not immunoreactive.

Interaction of AGE Immunoglobulins, AGE Albumin, or Patient-derived AGEs with Iron-loaded Lactoferrin Leads to Generation of ROIs—There is a close association between glycation and oxidation (2, 11, 12). Previous studies have shown that AGEs can produce ROIs (13, 14), a reaction which is enhanced in the presence of iron (14). In view of our observation that AGEs interact selectively with the iron binding protein LF (7), we set out to test the hypothesis that the association of AGEs with LF [illegible] might

target cells expressing AGE binding proteins. As a first step, we tested whether LF, appropriately loaded with iron (10), would promote AGE-mediated generation of ROIs. AGEs in the absence of LF generated only low levels of ROIs in the cytochrome *c* reduction assay (Fig. 2*A*, *AGE*). When iron-loaded LF was incubated with either AGE albumin prepared *in vitro* using glucose or glucose 6-phosphate or AGEs immunoisolated from diabetic plasma, generation of ROIs was enhanced (Fig. 2*A*, *AGE+LF*). This was due, almost entirely, to the formation of superoxide, as the increased signal in the cytochrome *c* assay was blocked by addition of superoxide dismutase (Fig. 2*A*, *AGE+LF+SOD*). Since similar results were obtained with each AGE preparation, further studies to assess the effect of LF on AGE-induced generation of ROIs were performed with AGE immunoglobulin and AGE albumin prepared *in vitro* with glucose 6-phosphate. When either AGE immunoglobulin (Fig. 2, *B* and *D*) or AGE albumin (Fig. 2, *C* and *D*) was incubated with LF, generation of ROIs occurred in a dose-dependent fashion: proportional to both the AGE and LF concentrations. In each case, the presence of LF enhanced ROI generation compared with AGEs alone (Fig. 2, *B* and *C*).

If enhanced generation of ROIs by AGE/LF required the binding and/or close association of these 2 molecules, we reasoned that antibodies which blocked this binding (anti-AGE IgG or anti-LF IgG) should prevent production of ROIs. Both

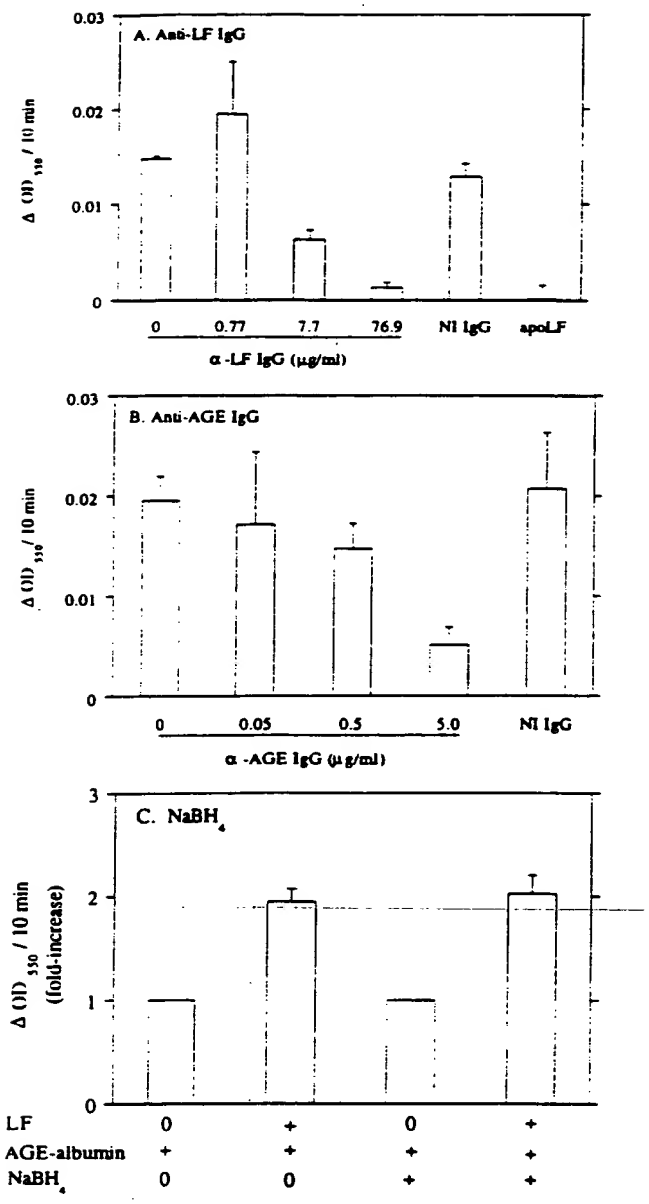

FIG. 3. **Effect of anti-LF IgG and anti-AGE IgG, and NaBH4 reduction on AGE albumin interaction with LF leading to generation of ROIs.** *A*. AGE albumin (300 nM) was incubated with LF (125 nM) alone or in the presence of the indicated concentration of anti-LF IgG or nonimmune IgG (*NI IgG*; 77 μg/ml). As indicated, LF was replaced with apolactoferrin (*apoLF*; 125 nM). Generation of ROIs was assessed in the cytochrome *c* reduction assay. *B*, the same experiment was performed as in *A* except that the indicated concentration of anti-AGE IgG was employed and NI IgG was 5 μg/ml. *C*, AGE albumin (300 nM) or AGE albumin pretreated by reduction with $NaBH_4$ (300 nM; prepared as described in the text) was incubated in buffer alone or in the presence of LF, and generation of ROIs was assessed. In each case, the mean ± S.E. of triplicate determinations is shown.

a dose-dependent manner (Fig. 3, *A* and *B*, respectively) and at antibody concentrations which also blocked the binding of AGEs to LF or LF-L (data not shown). In contrast, nonimmune IgG was without effect (Fig. 3, *A* and *B*, *NI IgG*). LF-enhanced generation of ROIs by AGEs required iron to be present in LF, as apolactoferrin was inactive (Fig. 3*A*, *apoLF*). Although it is possible that some residual iron passively bound to LF could be released into solution, where it could interact with AGEs, this was unlikely in view of the inhibitory effect of anti-AGE IgG and anti-LF IgG, the latter suggesting that a close association of AGEs to LF is necessary. This conclusion is supported by th results of our studies of AGE-induced oxidant stress in cell culture and *in vivo* (see below).

The glycated proteins employed in these experiments were likely to contain Schiff bases and Amadori products, as well as a range of advanced products. To determine if AGEs were involved in ROI generation by AGE albumin/LF mixtures, glycated albumin was subjected to sodium borohydride reduction (17). Following this treatment, AGE albumin still produced ROIs when incubated with iron-loaded lactoferrin, suggesting that advanced products contribute to oxygen free radical production (Fig. 3*C*).

Interaction of AGE Albumin with ECs Leads to Generation of Oxidant Stress—The results of the *in vitro* experiments with AGEs and iron-loaded LF above supported the hypothesis that AGE interaction with LF-L or LF could lead to the enhanced generation of ROIs. However, the relevance of these observations to the *in vivo* situation was not obvious, as LF in th plasma is minimally saturated with iron (36) and mechanisms through which AGEs could interact with ferric iron tightly bound to LF were unclear. Our goal was to test the hypothesis that AGEs, through their interaction with specific cell surface binding sites of which LF-L or LF is a component (7), generated cellular oxidant stress in culture and *in vivo*, thereby modulating cellular functions. Thus, an important next step was to determine if exposure of cultured ECs to AGEs in iron-free/serum-free medium would lead to the appearance of thiobarbituric acid reactive substances (TBARS) in the cells. Generation of TBARS occurred when cultured ECs were incubated with AGE albumin (note that AGEs are nonreactive in the TBARS assay), was dependent on the AGE albumin concentration (Fig. 4*A*, *AGE albumin*), and parallelled levels of AGE albumin which have been shown to result in occupancy of EC surface AGE binding sites (37). Levels of TBARS in control cultures exposed to native albumin were identical with results observed with ECs incubated in medium alone (Fig. 4*A*, *albumin*). Consistent with a role for binding of AGEs to endothelium for their induction of EC oxidant stress, generation of TBARS was blocked by either anti-RAGE IgG or anti-LF-L IgG (Fig. 4, *B* and *C*, respectively), both of which independently prevented binding of AGEs to the surface of ECs or mononuclear phagocytes (7, 9). Pretreatment of ECs with antioxidants, probucol, or *N*-acetylcysteine also blocked AGE-induced generation of TBARS, indicative of oxidant stress underlying TBARS formation (Fig. 4*D*). AGE albumin-induced EC oxidant stress was largely blocked on addition of either superoxide dismutase (200 units/ml), catalase, or glutathione peroxidase to the medium (Fig. 4*E*), suggesting the involvement of multiple species of oxygen-free radicals, including superoxide and hydrogen peroxide. The potential physiologic relevance of these observations with AGEs prepared *in vitro* was suggested by experiments in which ECs were exposed to AGEs immunoisolated from diabetic plasma; levels of TBARS in the cell monolayer increased (Fig. 4*F*).

Generation of ROIs by AGE albumin bound to the EC surface did not alter cell viability, based on continued trypan blue exclusion, lack of release of lactate dehydrogenase, continued adherence to the culture substrate, and subsequent growth on serial passage of the cells. This led us to consider if AGE-mediated generation of ROIs modulated cellular functions in a more subtle way. One such mechanism would be AGE-induced activation of the transcription factor NF-κB (30), which has been previously shown to be sensitive to oxidant stress (38). Although ECs exposed to native albumin demonstrated no induced gel shift band (Fig. 5*A*, *lane 2*), cultures incubated with AGE albumin showed activation of NF-κB, based on electro-

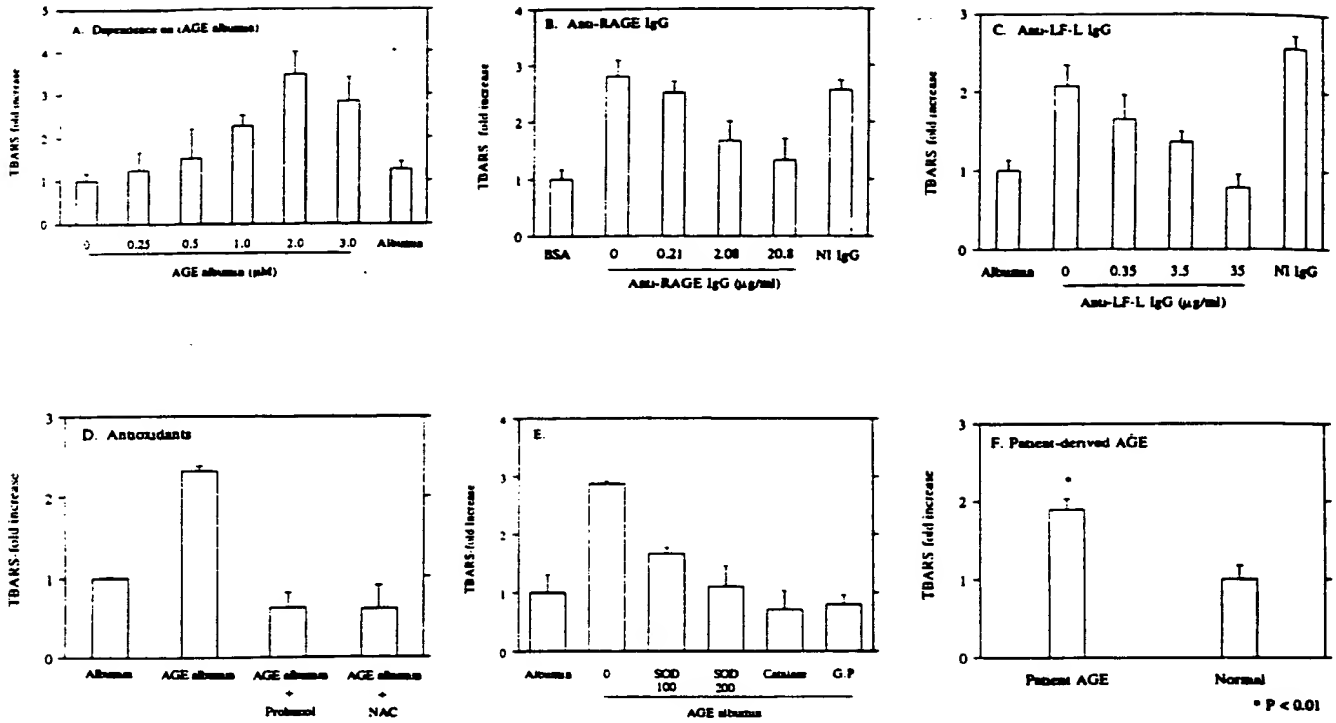

FIG. 4. **Incubation of AGE albumin with cultured endothelial cells (ECs) leads to oxidant stress: generation of TBARS.** *A*, AGE albumin at the indicated concentration was incubated with ECs (10^6 cells/well) for 18 h at 37 °C, and then the cell monolayer was processed to evaluate generation of TBARS. *B* and *C*, AGE albumin (1 μM) was incubated with ECs as above, and the indicated concentration of either anti-RAGE IgG (*B*), anti-LF IgG (*C*), or nonimmune IgG (*NI IgG; panel B*, 20 μg/ml, and *panel C*, 35 μg/ml) was added to the reaction mixture. Generation of TBARS was assessed. Albumin indicates ECs were incubated with nonglycated albumin (1 μM). *D*, AGE albumin (1 μM) was incubated with ECs pretreated with either probucol (50 μM) or *N*-acetylcysteine (30 mM), and generation of TBARS was assessed. *E*, AGE albumin (1 μM) was incubated with ECs alone or with cultures to which superoxide dismutase (100 or 200 units/ml), catalase (100 units/ml), and glutathione peroxidase (*GP*; 50 units/ml) had been added. Generation of TBARS was assessed. *F*, AGEs immunoisolated from the plasma of patients with diabetes (0.3 μM AGE albumin equivalents; $n = 8$) were incubated with ECs, and generation of TBARS was studied. *Normal* indicates eluate from the anti-AGE IgG column following application of the same volume of plasma from normal individuals ($n = 3$) was processed identically and applied to cells as above. In each case, the mean ± S.E. of triplicate determinations is shown.

phoretic mobility gel shift assays (EMSA; Fig. 5*A*, *lane 3*). The gel shift band observed in nuclear extracts from AGE- treated EC cultures co-migrated with that observed in cells incubated with TNF (Fig. 5*A*, *lanes 3* and *9*, respectively), a known inducer of NF-κB in ECs (39). Appearance of the AGE-induced band was blocked by a 100-fold excess of unlabeled NF-κB probe (Fig. 5*A*, *lane 10*), but not by an unrelated probe (Sp1; Fig. 5*A*, *lane 11*). AGE-induced activation of NF-κB was blocked completely when ECs were pretreated with antioxidants (*N*-acetylcysteine and probucol) (Fig. 5*A*, *lanes 7* and *8*), as well as receptor blockade with anti-RAGE IgG or anti-LF-L IgG (Fig. 5*A*, *lanes 4* and *5*), although the same amount of nonimmune IgG did not have a similar effect (Fig. 5*A*, *lane 6*). Taken together, these results indicate that binding of AGEs to the EC surface generates an oxidant stress which results in activation of NF-κB.

Infusion of AGEs into Normal Animals Leads to Generation of an Oxidant Stress—The most crucial test of our hypothesis concerning the potential of AGEs bound to their cell surface binding sites to generate oxidant stress was to perform experiments *in vivo* in normal animals in which plasma/tissue antioxidant mechanisms are intact. This led us to infuse AGE albumin into normal animals and to determine if TBARS formed in the tissues. Immunohistologic studies were performed with a murine monoclonal antibody which reacts with malondialdehyde epitopes (32), the production of which parallels formation of TBARS (AGE albumin was nonreactive with this antibody; [illegible] of lungs from rats infused with AGE 6*A*), compared with those infused with nonglycated albumin (Fig. 6*B*). Staining was seen in the endothelium, and, especially in subendothelial layers of the vessel wall, consistent with a previous study in which AGEs bound to ECs were, in part, transferred across the monolayer by transcytosis and released at the basal cell surface (37). Similar staining was seen in virtually every field examined. Exposure of lung tissue sections to nonimmune IgG from animals infused with AGE albumin resulted in no staining (Fig. 6*C*).

Infusion of AGE albumin increased levels of TBARS in a spectrum of organs, which was not observed in animals infused with native albumin (Fig. 7*A*). Increased levels of TBARS in tissues was blocked by preinfusion of either anti-LF-L IgG or anti-RAGE IgG, but was not affected by nonimmune (NI) IgG or antibody to thrombomodulin (Fig. 7*B*), an unrelated EC surface molecule (23). As might be expected from the results with cultured ECs, the antioxidants probucol and *N*-acetylcysteine blocked AGE-induced TBARS formation in the organs (Fig. 7*C*).

As an additional test of our hypothesis concerning AGE-mediated induction of oxidant stress *in vivo*, it was important to assess if consequences of cellular generation of ROIs were evident in organs from animals infused with AGEs, such as induction of heme oxygenase and activation of NF-κB. Increased expression of heme oxygenase mRNA has been associated with induction of oxidant stress *in vivo* (40). Northern blots of total RNA obtained from livers of mice infused with AGE albumin were hybridized with ^{32}P-labeled murine heme oxygenase

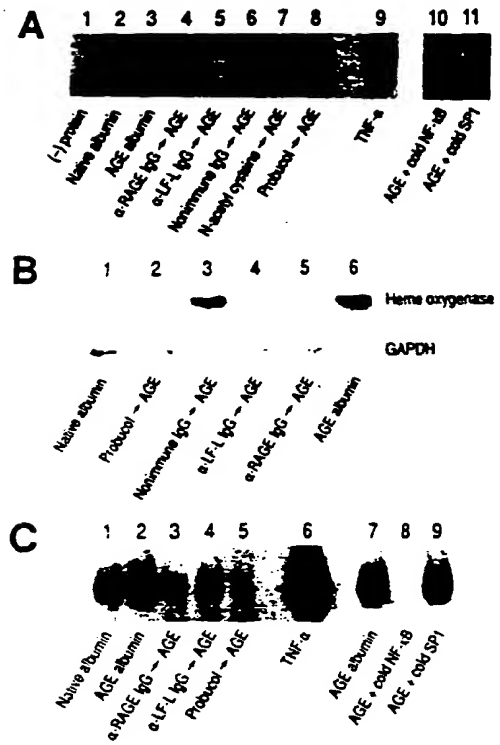

FIG. 5. **AGE-induced cellular oxidant stress: activation of NF-κB (*A* and *C*) and induction of heme oxygenase mRNA (*B*).** *A*, binding of nuclear proteins from cultured mouse ECs to the consensus murine NF-κB promoter sequence following exposure of cultures to AGE albumin: EMSA. The binding of nuclear proteins to the NF-κB site was demonstrated using EMSA. All lanes contained the labeled probe, and other additions are indicated below the numbered lane. *B*, induction of heme oxygenase mRNA in mice infused with AGE albumin. Mice were infused with AGE albumin or native albumin alone, or in the presence of probucol or antibodies to AGE binding proteins. Liver was harvested, RNA was extracted and subjected to electrophoresis, and blots were probed with radiolabeled cDNA probes for heme oxygenase or for glyceraldehyde phosphate dehydrogenase. Where indicated, mice were pretreated for 30 min with anti-RAGE (*α-RAGE IgG*), anti-LF-L IgG (*α-LF-L IgG*), nonimmune IgG, or probucol. Details of procedure are described in the text. *C*, activation of NF-κB in mice infused with AGE albumin. Mice were infused with either AGE albumin or native albumin, as above, livers were harvested, and nuclear extracts were prepared. Where indicated, mice were pretreated with either the antibodies or antioxidants as noted in the figure. The binding of nuclear proteins to the NF-κB site was demonstrated using EMSA. All lanes contained the labeled probe, and other additions are indicated below the numbered lane.

increased levels of heme oxygenase mRNA were observed in AGE-treated animals (Fig. 5*B*, *lanes 1* and *6*). Enhanced expression of heme oxygenase mRNA was prevented by pretreatment of mice with antibodies to the cellular AGE binding proteins, anti-LF-L IgG (Fig. 5*B*, *lane 4*) and anti-RAGE IgG (Fig. 5*B*, *lane 5*), but not with the same amount of nonimmune IgG (Fig. 5*B*, *lane 3*). In addition, the antioxidant probucol blocked AGE-induced increase in heme oxygenase mRNA (Fig. 5*B*, *lane 2*).

Enhanced activation of NF-κB was also observed in the livers of animals infused with AGE albumin (Fig. 5*C*). Gel shift assays using ^{32}P-labeled murine NF-κB probe showed a strong band in samples from animals infused with AGE albumin (Fig. 5*C*, *lanes 2* and *7*) *versus* no signal with nuclear extracts from animals infused with control/nonglycated albumin (Fig. 5*C*, *lane 1*). Pretreatment of animals with either antibodies to AGE binding proteins, anti-RAGE IgG, or anti-LF-L IgG greatly diminished NF-κB activation (Fig. 5*C*, *lanes 3* and *4*). NF-κB

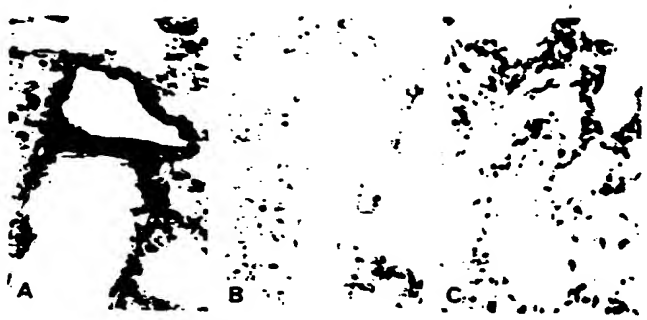

FIG. 6. **Immunohistologic detection of malondialdehyde determinants in the vasculature of rats infused with AGE albumin *versus* native albumin.** Rats were infused with AGE albumin (1 mg/animal; *A*) or native albumin (1 mg/animal; *B*); after 60 min, they were sacrificed, and tissues were processed for detection of malondialdehyde epitopes by indirect immunoalkaline phosphatase, as described in the text. In *panel C*, sections from an animal infused with AGE albumin were reacted with nonimmune IgG and then processed as in *A* and *B*. Magnification ×400.

activation in response to infused AGE albumin was also largely decreased in mice which had been pretreated with probucol (Fig. 5*C*, *lane 5*). The gel shift band observed in nuclear extracts from AGE-treated animals co-migrated with the major band observed in mice exposed to TNF (Fig. 5*C*, *lane 6*), and its appearance was blocked by excess unlabeled NF-κB probe (Fig. 5*C*, *lane 8*), but not by excess unlabeled Sp1 probe (Fig. 5*C*, *lane 9*).

DISCUSSION

Nonenzymatic glycation of proteins is closely linked to oxidative processes (2, 11, 12). The AGEs found in tissues which have been characterized to date are likely to have arisen by these mechanisms (2, 4). This is not surprising in view of the generation of oxygen-free radicals by glucose in aqueous solution, which, at high concentrations, has been shown to perturb endothelial cell function by a mechanism involving generation of ROIs (41).

The hypothesis underlying our study was that AGEs bound to cellular surfaces induce oxidant stress, thereby modulating cellular functions, even in the presence of intact anti-oxidant mechanisms. Our data support this concept, showing that AGE-mediated production of ROIs, whether by AGEs prepared *in vitro* or those immunoisolated from diabetic plasma, can generate an oxidant stress in target cells via interaction with specific AGE binding sites, RAGE and LF-L. In the endothelium we have demonstrated that a consequence of AGE-mediated ROI generation is activation of NF-κB, potentially modulating the expression of a host of cytokines and adherence molecules. It is likely that there are multiple consequences of this oxidant stress, as ECs undergo a spectrum of functional changes when they are exposed to oxidants. For example, ROIs could contribute to an AGE-induced increase in EC monolayer permeability (37). In addition, although there was no change in viability observed in cultures exposed to AGEs, by stressing cellular oxidant mechanisms, AGEs could sensitize the endothelium, thereby compromising the cellular response to other environmental stimuli. These results are not limited to ECs, as we have observed increased TBARS and activation of NF-κB in mononuclear phagocytes exposed to AGEs, which was also prevented by antibodies which block access to RAGE and LF-L or LF (data not shown). In a different context, the AGEs capable of inducing oxidant stress can themselves be cell surface-associated, as we have observed that red blood cells from patients with diabetes bind to the endothelium, via interaction with the specific AGE binding proteins, and induce an elevation of

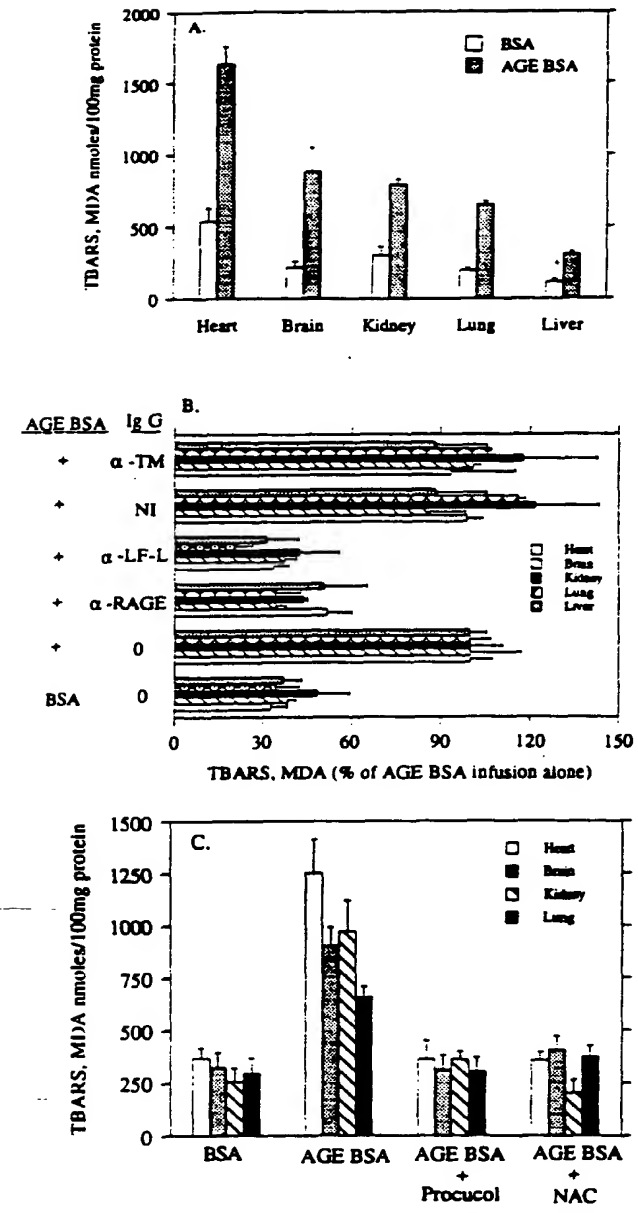

FIG. 7. **Generation of TBARS in mice infused with AGE albumin.** *A*, mice were infused with either AGE albumin (*AGE BSA*; 100 μg/animal; intravenous) or native albumin (*BSA*; 100 μg/animal; intravenous). Organs were harvested 60 min later for assessment of the formation of TBARS. *B*, mice were infused with AGE albumin as above, except, as indicated, animals were pretreated with anti-RAGE IgG (*α-RAGE*; 40 μg/animal; intravenous), anti-LF-L IgG (*α-LF-L*; 40 μg/animal; intravenous), anti-thrombomodulin IgG (*α-TM*; 40 μg/animal; intravenous), or nonimmune IgG (*NI*; 40 μg/animal; intravenous). The indicated organs were harvested 60 min later for determination of TBARS. *C*, mice were infused with AGE albumin (*AGE BSA*) as above, except, as indicated, animals were pretreated with probucol (50 μM; intravenous) or *N*-acetylcysteine (30 mM; intravenous). In each case, the mean ± S.E. of triplicate determinations is shown. Samples from animals infused with native albumin are indicated as BSA in *panels A–C*.

TBARS and activation of NF-κB (42). Thus, diabetic red cells bearing AGEs on their surfaces have the capacity to perturb vascular function in the micro- and macrovasculature.

The potential relevance of AGEs as an oxidizing stimulus is suggested by the generation of TBARS, as well as the enhancement of both the activation of NF-κB and increased levels of heme oxygenase mRNA in normal mice infused with AGE albumin. The localizati n of malondialdehyde determinants to the vessel wall just after infusion of AGEs into rats is consistent with the concept that AGEs in the intravascular space interact with endothelium, potentially altering vascular function. In the presence of the potent and multifaceted antioxidant capacity of normal plasma and tissues, AGEs still led to induction of oxidant stress. Of note is that AGE-induced formation of TBARS in normal mice was short-lived, as TBARS had disappeared within 2–3 h of the AGE infusion. This suggests the importance of the intact antioxidant mechanisms in normal animals for limiting oxidant stress. Consistent with this hypothesis, pretreatment of mice with the glutathione peroxidase inhibitor diethylmaleic acid (43) led to enhanced TBARS generation on subsequent infusion of AGE albumin (data not shown). In patients with diabetes in which antioxidant capacity has been shown to be diminished (44–46), AGE-induced oxidant stress could be amplified. The association of increased levels of TBARS in the plasma of patients with diabetic complications supports a relationship between oxidative processes and end-organ compromise in this context (46).

These data provide a first step linking the well-known oxidant properties of AGEs to induction of oxidant stress in cells bearing AGE receptors. However, many questions remain unanswered. For example, do the cell surface AGE binding proteins, RAGE and LF-L, simply tether an oxidizing stimulus, AGEs, to the cell, or do they have a more active role in AGE-mediated generation of oxygen-free radicals? Our data show that interaction of AGEs with their cell surface receptors is a critical link in AGE-induced oxidant stress. Furthermore, the studies in which superoxide dismutase, catalase, and glutathione peroxidase (which would not be expected to gain access to the cytosol) blocked induction of oxidant stress suggest that AGE-mediated generation of ROIs occurs at the cell surface or in a pool of endocytosed ligand to which the added oxygen radical scavengers had access. Our data do not distinguish between the latter possibilities, although they do indicate that it is unlikely for AGE-induced oxidant stress to originate in the cytosol. In view of the capacity of AGEs interacting with iron-loaded LF to produce ROIs *in vitro*, it would be relevant to know the extent to which cell surface LF-L is saturated with ferric iron and if AGEs interact with this ion facilitating oxygen-free radical generation.

Thus, although the detailed mechanisms of formation and characterization of ROI species generated by AGEs have not yet been defined, these studies indicate that AGEs induce oxidant stress *in vivo* and *in vitro*, a process involving cell surface AGE binding proteins and likely to have far ranging effects on end-organ function. Additional studies will be required to delineate the mechanisms involved and to determine if this pathway underlies the myriad cellular properties perturbed during the interaction of AGEs with endothelial cells, monocytes, and other cells bearing receptors/binding proteins for AGEs.

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